

Histone Hyperacetylation Induced by Histone Deacetylase Inhibitors Is Not Sufficient to Cause Growth Inhibition in Human Dermal Fibroblasts*

Received for publication, January 10, 2001, and in revised form, March 23, 2001
Published, JBC Papers in Press, April 13, 2001, DOI 10.1074/jbc.M100206200

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Use of specific histone deacetylase inhibitors has revealed critical roles for the histone deacetylases (HDAC) in controlling proliferation. Although many studies have correlated the function of HDAC inhibitors with the hyperacetylation of histones, few studies have specifically addressed whether the accumulation of acetylated histones, caused by HDAC inhibitor treatment, is responsible for growth inhibition. In the present study we show that HDAC inhibitors cause growth inhibition in normal and transformed keratinocytes but not in normal dermal fibroblasts. This was despite the observation that the HDAC inhibitor, suberic bishydroxamate (SBHA), caused a kinetically similar accumulation of hyperacetylated histones. This cell type-specific response to SBHA was not due to the inactivation of SBHA by fibroblasts, nor was it due to differences in the expression of specific HDAC family members. Remarkably, overexpression of HDACs 1, 4, and 6 in normal human fibroblasts resulted in cells that could be growth-inhibited by SBHA. These data suggest that, although histone acetylation is a major target for HDAC inhibitors, the accumulation of hyperacetylated histones is not sufficient to cause growth inhibition in all cell types. This suggests that growth inhibition, caused by HDAC inhibitors, may be the culmination of histone hyperacetylation acting in concert with other growth regulatory pathways.

Analysis of histone-modifying enzymes such as the histone acetyltransferases (HATs)¹ and deacetylases has resulted in

significant advances in our understanding of transcriptional regulation (1–4). These studies have resulted in a model of transcription in which transcriptionally competent genes are transcribed or repressed dependent upon their ability to recruit either HATs or histone deacetylases to the promoter (4). In these models, recruited histone acetyltransferases associate with transcription factor complexes (5–8), resulting in the acetylation of nucleosomal histones, relaxation of nucleosomal integrity, and hence transcription. Conversely, transcriptional repression occurs when histone deacetylases (and cofactors) are recruited to DNA-bound transcription factors, resulting in the removal of acetyl groups from NH₂-terminal lysines causing a “tightening” of nucleosomal integrity and a suppression of transcription (9–13).

The isolation and synthesis of new and potent inhibitors of histone deacetylase enzymes (HDACs) has allowed us to identify some of the biological outcomes resulting from manipulation of histone deacetylase activity. For example, it is now established that treatment of cells *in vitro* and *in vivo* with HDAC inhibitors can result in specific functional outcomes such as cell cycle arrest (14–16), apoptosis/cell death (17–19), or differentiation (19–21). These outcomes to a large extent are cell type-specific and have raised the potential that the HDAC inhibitors may represent a new and important class of anticancer therapeutic agents (4).

HDAC inhibitors comprise a diverse range of unrelated compounds that all induce an accumulation of hyperacetylated histones (21). The biological effects of these compounds (*e.g.* cell cycle arrest, cell death, or differentiation) are thought to result from the accumulation of acetylated histones and transcriptional activation that results from the use of these compounds. For instance, sodium butyrate (NaB), suberic bishydroxamate (SBHA), and trichostatin A (TSA) are all HDAC inhibitors that induce an accumulation of acetylated histone H4 (21, 22). These agents are also able to induce growth inhibition, which has been shown to be associated causally with an induction of the cyclin-dependent kinase inhibitor p21 in colon carcinoma cells (15).

Although the data for the colon carcinoma cells used in the

* This work was supported in part by Queensland Cancer Fund Grant 98/QCFN001G, a grant from the Princess Alexandra Hospital Foundation, a grant from the Australian Government Employees Medical Defense Fund, Australian National Health and Medical Research Committee Grant 142906, and a grant from the Garnett Passe and Rodney Williams Memorial Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[‡] Supported by an Australian postgraduate award.

[¶] Supported by a postgraduate scholarship awarded by the Garnett Passe and Rodney Williams Memorial Foundation.

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¹ The abbreviations used are: HAT, histone acetyltransferase; HDAC,

histone deacetylase; SBHA, suberic bishydroxamate; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human epidermal keratinocyte; HDF, human dermal fibroblast; R-PB, *R* enantiomeric form of phenylbutyrate; S-PB, *S* enantiomeric form of phenylbutyrate; AAHA, azelaic-1-hydroxyamate-9-anilide; HMBA, hexamethylene bisacetamide; TSA, trichostatin A; TUNEL, terminal dUTP nick-end labeling.

p21 studies appear compelling, there are several pieces of evidence that suggest that the biological outcomes, in response to the HDAC inhibitors in other cell types, may not be explained simply by an accumulation of acetylated histones. For instance, 1) it has been shown that certain cell types (human dermal fibroblasts or murine erythroleukemia cells) are able to grow in the presence of HDAC inhibitors and in the presence of hyperacetylated histones (6, 18, 22). 2) We have shown previously that the accumulation of acetylated histone H4 in response to NaB or TSA in keratinocytes and the squamous cell carcinoma cell line, SCC25, is transient and is temporally uncoupled from the process of growth inhibition (21). 3) Recently it has been suggested that the cell cycle regulator, Rb, mediates its growth inhibitory effects by associating with an HDAC and thus suppressing E2F activity (23–25). Although this has been convincingly shown *in vitro*, it should be noted that the E2F activation caused by HDAC inhibitors (23–25) is inconsistent with the growth inhibition observed in cells following HDAC inhibitor treatment. 4) Recent studies have shown that nonhistone proteins such as E2F1 (26) and p53 (27, 28) are subject to both acetylation and deacetylation, and more importantly these modifications result in alterations in functional activity of these important transcriptional controllers of growth and differentiation. 5) HDAC inhibitors do not lead to global deregulation of transcription (29). These observations raise the possibility that biological effects of HDAC inhibitors may be, in part, mediated by events independent of the acetylation of histones (30).

In the present study we wished to examine the relationship between the accumulation of acetylated histones in response to HDAC inhibitors and the induction of growth arrest in keratinocytes, fibroblasts, and SCC25 cells. We report that the induction of acetylated histones in fibroblasts is not associated with growth inhibition. Our data are consistent with a model in which growth inhibition in response to HDAC inhibitors is cell type-specific and dictated by the activity of pre-existing regulatory pathways in the cells.

MATERIALS AND METHODS

Cells, Tissue Culture, and Reagents—Human epidermal keratinocytes (HEKs) were isolated from neonatal foreskins following circumcision and cultured in keratinocyte serum-free medium (Life Technologies Inc, Sydney, New South Wales (NSW), Australia) as described (31). Human dermal fibroblasts (HDFs) were also isolated from foreskin samples following circumcision and were cultured in Dulbecco's modified Eagle's medium as described (32). The keratinocyte-derived squamous cell carcinoma cell line, SCC25, was obtained from the American Type Tissue Culture Collection and cultured as described (31). All cells in this study were used under subconfluent, proliferative conditions. The histone deacetylase inhibitors sodium butyrate (NaB), the R (R-PB) and S (S-PB) enantiomeric forms of phenylbutyrate, and the hybrid polar compound hexamethylene bisacetamide (HMBA) were purchased from Sigma (Sydney, NSW, Australia). The synthesis of azelaic-1-hydroxyaminate-9-anilide (AAHA) has been described previously (18) as has the synthesis of suberic bishydroxamate (SBHA, Ref. 33).

Plasmids, Transfections, and Cell Line Selection—Human histone deacetylase 1 (HDAC1; Ref. 34) and histone deacetylases 4, 5, and 6 (HDACs 4–6; Ref. 35) in the pBJ5 expression plasmid were a generous gift from Prof. Schreiber (Harvard University, Cambridge, MA). A glutathione *S*-transferase-tagged murine HDAC2 plasmid (36) and a human HDAC3 expression plasmid (in the pGEX expression plasmid; Ref. 37) were a generous gift from Prof. Seto (H. Lee Moffitt Cancer Center and Research Institute, University of San Francisco, San Francisco, CA). To establish stably expressing HDF cell lines, 75-cm² flasks of passage 2 HDFs (50% confluent) were transfected with 9 μ g of HDAC expression vector + 3 μ g of the pSV2neo neomycin expression vector followed by selection with 500 μ g/ml G418 (31). Transfection used a similar protocol to that described for keratinocytes (38) in the presence of LipofectAMINE Plus reagent as per manufacturer's instructions (Life Technologies, Inc.).

Apoptosis Assay—Estimates of apoptosis induced by NaB (3 mM), AAHA (3 μ M), or SBHA (100 μ M) were determined following a 24-h treatment with the compounds. Apoptosis was estimated using the

TUNEL assay as per the manufacturer's protocol (Roche, Brisbane, Queensland (QLD), Australia). Briefly, cells were cultured in 25-cm² flasks, trypsinized, and then subjected to cytospinning (100 \times *g* for 2 min) before being used in the TUNEL assay. An apoptotic index was then derived (apoptotic cells/total cells \times 100/1).

Western Blotting and Histone Isolation—Protein expression and histone acetylation status were determined by immunoblotting. For Rb, p21, acetyllysine, or FLAG-tagged HDAC protein analysis, cells were trypsinized and rinsed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄). An aliquot was removed for protein determination, and the remaining protein solubilized in 2 \times sample buffer (25 mM Tris-Cl, 5% glycerol, 5% SDS, 5% β -mercaptoethanol, 0.005% bromophenol blue) and boiled for 5 min. Twentyfive micrograms of protein were then run on a 7.5% SDS-PAGE gel (16 cm \times 18 cm), transferred to polyvinylidene difluoride membrane, and probed with antibodies specific for Rb (sc-50; 1:1000), p107 (sc-318; 1:1000), p130 (sc-317; 1:1000), p21 (sc-397; 1:1000; Santa Cruz Biotechnology, Brisbane, QLD, Australia), acetyllysine (06-933; 1:1000; Upstate Biotechnology, Parkville, Victoria, Australia), or anti-FLAG antibody (1 μ g/ml; F-3165; Sigma, Sydney, NSW, Australia). To determine histone acetylation status, cells were harvested by trypsinization and histones isolated as described (21). Five micrograms of purified histones were then run on a 15% SDS-PAGE gel and blotted to PVDF membrane and probed with either an antibody specific for acetylated histone H4 (06-598) or an antibody specific for acetylated lysines (06-933; Upstate Biotechnology Inc, Melbourne, Victoria, Australia). Purified H2A, H3, and H4 (Roche) were run in separate lanes to confirm the identity of the purified histones. All immunoblots were visualized using a primary antibody dilution of 1:1000 and chemiluminescent detection (ECL; Amersham, Sydney, NSW, Australia) as described (21). Quantitation of acetylation level was determined by densitometric analysis of the autoradiographs as described (21).

RNA Isolation and Northern Blotting—Total cellular RNA was isolated from cells following disruption in Trizol (Life Technologies, Inc.) using previously described protocols (38, 39). Enrichment for poly(A)⁺ RNA by oligo(dT)-cellulose (Collaborative Research Inc., Bedford, MA) chromatography was then performed as described (40). One microgram of poly(A)⁺ RNA (HEKs and HDFs) was then electrophoresed, transferred to nylon membrane (Hybond N; Amersham Pharmacia Biotech), and hybridized with probes specific for HDACs 1–6 and actin. Probes for specific HDACs were made from restriction digests of the expression plasmids using the following restriction enzymes: HDAC1 = *NotI*/*EcoRI*, HDAC2 = *XhoI*, HDAC3 = *EcoRI*, HDAC4 = *SalI*/*SacI*, HDAC5 = *SacI*/*SacII*, HDAC6 = *SphI*/*AvrII*. All inserts were gel-purified. Probe labeling with [³²P]dCTP (Geneworks, Adelaide, South Australia, Australia), hybridizing, and washing were performed as described previously (41). A probe against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize for loading inequalities and has been described previously (42). All blots were visualized by autoradiography with Kodak XAR5 film. Images were then captured using a laser densitometer (Molecular Dynamics, Sydney, NSW, Australia) and imported into Adobe Photoshop.

Proliferation Assay and Cell Cycle Analysis—Proliferation was measured directly either by assaying for [³H]thymidine incorporation (43) or by an analysis of the cell cycle in propidium iodide-stained cells as described (44).

Kinetic Analysis and Statistics—Dose response analysis for proliferation suppression by histone deacetylase inhibitors was estimated by nonlinear regression analysis with the following equation $P = P_0 e^{-k[I]}$, where P = DNA synthesis, P_0 = DNA synthesis in the absence of inhibitor, k = rate constant defining the inhibition DNA synthesis, and $[I]$ = the concentration of HDAC inhibitor (39). Dose-response analysis for the accumulation of acetylated histones was estimated by nonlinear regression analysis using the following equation: $Ac = (Ac_{max} * EC_{50}) / (EC_{50} + [I])$ where Ac = the acetylation level, Ac_{max} = the maximal amount of histone acetylation, EC_{50} = the concentration at which acetylation is half-maximal and $[I]$ = the concentration of HDAC inhibitor. All data were fitted using GraphPad Prism software (Brisbane, QLD, Australia).

RESULTS

Growth Inhibition by Histone Deacetylase Inhibitors Is Cell-selective—We have shown previously that the histone deacetylase inhibitors NaB and TSA are able to inhibit DNA synthesis in keratinocytes and a number of keratinocyte-derived squamous cell carcinoma cell lines (21). Treatment of keratinocytes and SCC25 cells with varying concentrations of NaB, R-PB,

TABLE I

IC₅₀ values for various histone deacetylase inhibitors for the inhibition of growth in keratinocytes or SCC25 cells

Proliferating keratinocytes (HEK) or squamous cell carcinoma cells (SCC25) were treated with varying concentrations of the indicated compounds for 24 h. In the final 3 h of treatment, cells were co-incubated with 2.5 μ Ci/ml [³H]methyl thymidine. DNA synthesis was then estimated and the IC₅₀ determined by nonlinear least squares regression analysis. Data are presented as the IC₅₀ value \pm standard error of the estimate.

	HEK	SCC25
NaB	0.26 \pm 0.11 mM	1.28 \pm 0.24 mM
R-PB		
S-PB		
AAHA	0.68 \pm 0.21 μ M	3.9 \pm 3.8 μ M
SBHA	11.7 \pm 8.46 μ M	5.39 \pm 5.6 μ M
HMBA		

S-PB, AAHA, SBHA, or the hybrid polar compound, HMBA, indicated that only NaB, AAHA, and SBHA induced growth inhibition (Table I). Dose-response analysis indicated that NaB and AAHA were slightly more potent at inducing growth arrest in keratinocytes than in SCC25 cells (Table I). In contrast, the EC₅₀ value for the inhibition of DNA synthesis by SBHA in HEKs and SCC25 cells was similar (Table I). A dose-response profile for the inhibition of DNA synthesis by R-PB or S-PB in HEKs and SCC25 cells was not possible despite analysis out to 6 mM. A modest inhibition of DNA synthesis with R-PB and S-PB was noted only at the 6 mM concentration in HEKs (R-PB, 40 \pm 13%; and S-PB, 53 \pm 24% that of untreated controls) and SCC25 cells (R-PB, 112 \pm 14%; and S-PB, 122 \pm 6% that of untreated controls). In contrast, these agents were not able to inhibit DNA synthesis in HDFs (Table II). In fact, NaB and AAHA, at high concentration, significantly increased DNA synthesis in the HDFs (Table II). However, growth inhibition of HDFs can eventually be induced by prolonged exposure (48 h) to high concentrations of SBHA (300 μ M). Furthermore, the insensitivity of HDFs to SBHA could be altered by changing media composition (data not shown). Combined, these data clearly show that keratinocytes and the keratinocyte-derived carcinoma cell line are sensitive to the growth inhibitory properties of histone deacetylase inhibitors whereas fibroblasts remain insensitive under similar conditions.

Cell cycle analysis of keratinocytes treated with varying concentrations of SBHA for 24 h indicated that the decrease in DNA synthesis observed in these cells (Table I) was associated with an accumulation in G₀/G₁ phase (Table III). Consistent with the results of the DNA synthesis assay in HDFs, the cell cycle analysis did not reveal any specific blockade of the cell cycle (Table III). There was a decrease in the percentage of HDFs in G₂/M phase following treatment with 100 μ M SBHA, which suggests that SBHA may reduce transit time through G₂/M phase and may be consistent with the modest increase in DNA synthesis observed in HDFs following SBHA treatment (Table II). Interestingly, the cell cycle profile observed in SCC25 cells treated with 100 μ M SBHA showed an accumulation in the G₂/M phase with a lesser, if any, accumulation of cells in G₀/G₁ (Table III). This contrasts with the findings observed in HEKs and suggests that different mechanisms of action may be invoked by SBHA treatment of keratinocytes and transformed keratinocytes. It should be noted that, following treatment with SBHA, ~30–50% of SCC25 became detached, which is consistent with cell death. A similar result has been described previously for these cells (44). Thus, the findings in the SCC25 cells most likely represent a combination of a G₂/M accumulation of SBHA-treated cells and the induction of cell death.

A well characterized target of HDAC inhibitor action is the

TABLE II

Ability of the histone deacetylase inhibitors to inhibit DNA synthesis in keratinocytes and fibroblasts

Proliferating keratinocytes (HEK) or human dermal fibroblasts (HDF) were treated with maximal concentrations of the indicated compounds for 24 h. In the final 3 h of treatment, cells were co-incubated with 2.5 μ Ci/ml [³H]methyl thymidine. DNA synthesis was then estimated and the inhibition of DNA synthesis expressed as a percentage of the value obtained in the untreated cells. The data is presented as mean \pm S.E. of triplicate determinations from two independent experiments. * Denotes significant difference ($p < 0.05$) determined by Student's t test.

	HEK	HDF
Untreated	100 \pm 3.5	100 \pm 5
NaB (3 mM)	1 \pm 0.2*	186 \pm 27*
R-PB (6 mM)	40 \pm 13*	171 \pm 47
S-PB (6 mM)	53 \pm 24	94 \pm 23
AAHA (3 μ M)	1.5 \pm 0.4*	151 \pm 13*
SBHA (100 μ M)	1.2 \pm 0.3*	164 \pm 43
HMBA (1 mM)	202 \pm 79	93 \pm 20

TABLE III

Cell cycle analysis on human dermal fibroblasts, SCC25 cells, and human epidermal keratinocytes treated with varying concentrations of SBHA

Proliferating cells were treated with varying concentrations of SBHA for 24 h. Cells were then trypsinized and stored in 70% ethanol at -20° C until used for cell cycle analysis. Data are presented as percentage of total cells present in the different cell cycle phases.

[SBHA]	G ₀ /G ₁	S	G ₂ /M
Human dermal fibroblasts			
0 μ M	49.9%	20.9%	29.2%
1 μ M	49.0%	23.3%	27.7%
3 μ M	47.6%	22.3%	30.1%
10 μ M	45.7%	24.2%	30.0%
30 μ M	53.3%	20.2%	26.4%
100 μ M	56.6%	24.1%	19.3%
Human epidermal keratinocytes			
0 μ M	60.5%	21.9%	17.6%
1 μ M	56.1%	29.6%	14.3%
3 μ M	57.1%	27.8%	15.1%
10 μ M	64.0%	16.1%	20.0%
30 μ M	64.6%	14.5%	20.9%
100 μ M	71.4%	7.2%	21.4%
SCC25 cells			
0 μ M	44.0%	43.5%	12.5%
1 μ M	41.6%	43.8%	14.6%
3 μ M	42.6%	41.2%	16.2%
10 μ M	40.5%	45.1%	14.3%
30 μ M	40.0%	43.1%	16.9%
100 μ M	45.5%	32.5%	22.0%

induction of p21 (15) and/or the accumulation of hypophosphorylated Rb (16), both of which are thought to be mediated by alterations in transcriptional control mediated by HDACs (45–47). Following a 24-h treatment of HEKs, HDFs, and SCC25 cells with SBHA (100 μ M), there was an induction of p21 protein expression in all the cell types (Fig. 1). However, the induction of p21 was associated with a corresponding alteration in the phosphorylation status of the pocket proteins Rb or p107 only in keratinocytes but not in HDFs or growth-inhibited SCC25 cells (Fig. 1). Moreover, significant levels of expression of the pocket protein p130 were observed only in keratinocytes but were not altered by SBHA treatment (Fig. 1). The lack of an association between SBHA-induced growth inhibition and the phosphorylation status of Rb and p107 in SCC25 cells suggests that a mechanism distinct from E2F suppression may be involved in SBHA-induced growth suppression in SCC25 cells. These findings are consistent with the cell cycle analysis (Table III), which indicated that SBHA selectively induced an accumulation of SCC25 cells in the G₂/M phase and selectively induced an accumulation of keratinocytes in the G₀/G₁ phase. Combined, these data show that the ability of SBHA to induce

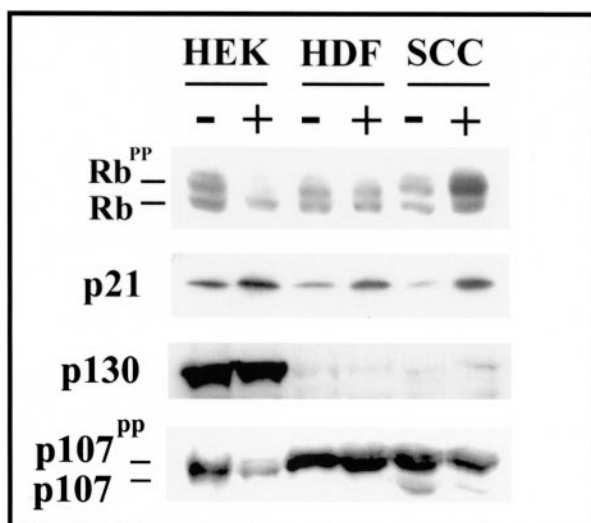


FIG. 1. Pocket protein (Rb, p107, p130) and p21 protein expression in HEKs, HDFs, and SCC25 cells in response to SBHA. Keratinocytes (HEK), fibroblasts (HDF), and SCC25 cells (SCC) were left untreated (–) or were treated with 100 μ M SBHA for 24 h (+). Twenty-five μ g of cellular protein were electrophoresed, blotted, and then probed for the expression of Rb, p21, p130, and p107. Hyperphosphorylated forms of Rb (Rb^{PP}) and p107 ($p107^{PP}$) as well as hypophosphorylated forms of Rb and p107 are shown.

growth inhibition may be mediated by events in G_0/G_1 or G_2/M dependent upon the cell type or transformation status.

An alternative explanation for the decrease in DNA synthesis induced by HDAC inhibitors in keratinocytes and SCC25 cells may be attributable to apoptosis. Previously, agents such as NaB or TSA have been reported to induce apoptosis in colon carcinoma cell lines (48) and C3H10T1/2 cells (19). Furthermore, it has been shown that ABHA treatment of SCC25 cells is associated with detachment and cell death (44). In the present study, we found no evidence of apoptosis, by TUNEL assay, in adherent HEKs and SCC25 cells following NaB, AAHA, or SBHA treatment (data not shown). However, NaB, AAHA, and SBHA treatment did cause a significant proportion of the adherent SCC25 cells to lift off the culture dish. These data are consistent with a non-apoptotic/necrotic mechanism of cell death in SCC25 cells (data not shown). These data indicate that the decrease in DNA synthesis observed in the adherent HEKs following HDAC inhibitor treatment was not due to apoptosis or cell death, whereas the G_2/M phase accumulation, observed in SCC25 cells, may be a prelude to detachment and cell death.

Histone Deacetylase Inhibitors Induce an Accumulation of Acetylated Histone H4 in HEKs, HDFs, and SCC25 Cells—The simplest explanation for the resistance of fibroblasts to the histone deacetylase inhibitors is that the inhibitors are unable to induce an accumulation of acetylated histones due to the inactivity of these compounds in fibroblasts. We have shown previously that accumulation of acetylated histones in keratinocytes and SCC25 cells is transient and is maximal at 8 h in response to NaB or TSA. Therefore, we treated HEKs, HDFs, and SCC25 cells with the various HDAC inhibitors for 8 h and then isolated the histone proteins from these cells. A Western blot detecting acetylated histone H4 is shown in Fig. 2. These data show that NaB, SBHA, and AAHA are able to induce an hyperacetylation of histone H4 in all cell types (H4 acetylation caused by AAHA is visible in HDFs with a longer gel exposure time). These data also show that the inability of the HDAC inhibitors to cause growth inhibition of HDFs is not due to an inability to induce histone H4 acetylation (a similar result was noted with H3 acetylation; data not shown). It is also of interest to note that HMBA, R-PB, and S-PB did not induce an accu-

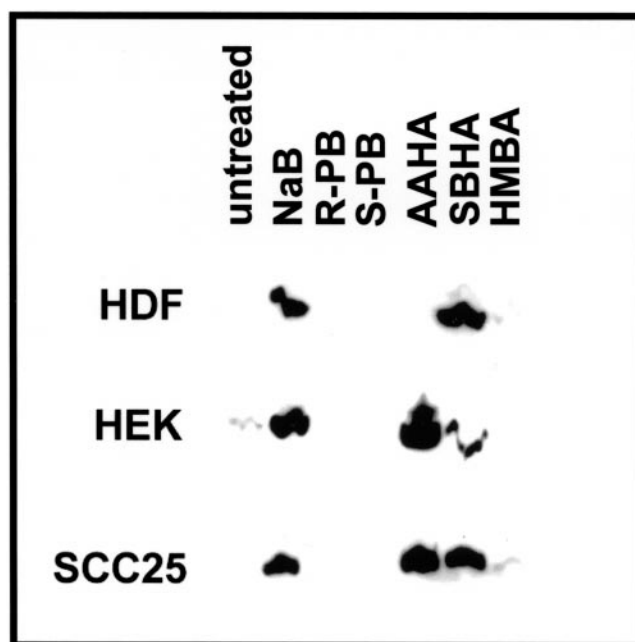


FIG. 2. Accumulation of acetylated histone H4 in response to HDAC inhibitors. Histones were purified from cultured human epidermal keratinocytes (HEK), human dermal fibroblasts (HDF), or SCC25 cells (SCC25) following treatment with potential HDAC inhibitors for 8 h (lanes 2–7). Five μ g of purified histone protein were then run on a 15% SDS-PAGE gel, blotted, and probed with an antibody specific for acetylated histone H4. NaB = 3 mM; R-PB = 6 mM; S-PB = 6 mM; AAHA = 3 μ M; SBHA = 100 μ M; HMBA = 1 mM.

mulation of acetylated H4 and were unable to produce significant growth arrest in any of the cells.

The inability to induce growth arrest in the HDFs, in response to SBHA, was examined in more detail to determine whether there were differences in the kinetics for the inhibition of HDACs that may explain the inability of SBHA to cause growth inhibition of HDFs (Fig. 3). Dose-response analysis for the inhibition of growth (Fig. 3A) and the accumulation of acetylated histone H4 (Fig. 3B) was examined in HEKs and HDFs. SBHA induced growth inhibition in HEKs with an IC_{50} value of $11.7 \pm 8.5 \mu$ M (Fig. 3A). Similarly, SBHA induced an accumulation of acetylated histone H4 in HEKs with an EC_{50} value $7.9 \pm 7.8 \mu$ M. In contrast, HDFs were resistant to the growth inhibitory properties of SBHA (Fig. 3A) but accumulated acetylated histone H4 with an EC_{50} value similar to that for HEKs ($1.0 \pm 1.8 \mu$ M). These data clearly show that, although HDFs are resistant to the growth inhibitory properties of SBHA, both HEKs and HDFs are sensitive to the HDAC inhibitory action of SBHA.

An explanation for the resistance of HDFs to SBHA-mediated growth inhibition could be due to the degradation/metabolism of SBHA by HDFs or HDF media. This possibility was formally tested by taking media from HDFs treated with SBHA for 24 h and placing it on SCC25 cells for an additional 24 h (Fig. 3C). Media from HDFs treated with SBHA (300 μ M) for 24 h was able to cause growth inhibition in SCC25 cells of similar magnitude to that of SCC25 cells treated directly with SBHA (Fig. 3C). This observation clearly indicates that SBHA is not selectively inactivated by HDFs and suggests that another explanation for the growth inhibitor resistance of HDFs exists. This may include the possibility that 1) the HDAC family member (e.g. HDACs 1–8; Refs. 34–52) responsible for mediating growth inhibition, in response to SBHA, may be absent or poorly expressed in fibroblasts compared with keratinocytes or SCC cells; 2) the growth inhibitory action of the HDAC inhibitors may be mediated by the hyperacetylation of

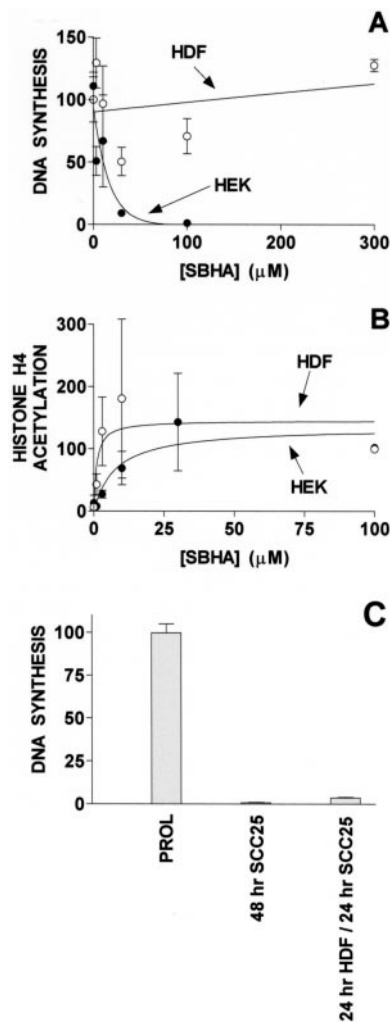


FIG. 3. Kinetics of growth suppression and histone H4 acetylation of HEKs and HDFs in response to varying concentrations of SBHA. Proliferating keratinocytes (HEK) or dermal fibroblasts (HDF) were treated with varying concentrations of SBHA. DNA synthesis in response to a 24-h treatment with varying concentrations of SBHA was then estimated by thymidine incorporation (A). For DNA synthesis cells were incubated with 2.5 μ Ci of [3 H] thymidine for 3 h, followed by analysis of dpm of thymidine/ μ g of cellular protein. Data are presented as mean \pm S.E. of triplicate determinations from two experiments and expressed as a percentage of the value of the untreated cells. The line represents the line of best fit determined by nonlinear regression analysis (HEK; \bullet) or linear regression analysis (HDF; \circ). B, following an 8-h treatment of HEKs and HDFs with varying concentrations of SBHA, histone proteins were purified and 5 μ g were used in a Western blot to determine histone H4 acetylation. Autoradiograms were scanned by laser scanning densitometer and data fitted by nonlinear regression analysis. Data for keratinocytes (HEK; \bullet) and fibroblasts (HDF; \circ) represent mean \pm S.E. of at least four independent experiments. Histone acetylation is presented as arbitrary units. C, DNA synthesis was measured in untreated proliferating SCC25 cells (PROL) or in SCC25 cells treated with 300 μ M SBHA for 48 h (48 hr SCC25). Alternatively, HDFs were treated with 300 μ M SBHA for 24 h and the media removed and placed on SCC25 cells for another 24 h (24 hr HDF / 24 hr SCC25). Data are presented as mean \pm S.E. of triplicate determinations.

non-histone proteins; or 3) the HDAC complexes involved in SBHA-induced growth inhibition may be cell type-specific or gene-specific.

HEKs and HDFs Have a Different Complement of HDAC mRNA Expression—The lack of an association between the hyperacetylation of histones and the inhibition of growth of HDFs, in response to HDAC inhibitors, may be attributable to cell type-specific expression of different HDAC family members. We therefore screened the HEKs, HDFs, and SCC25 cells

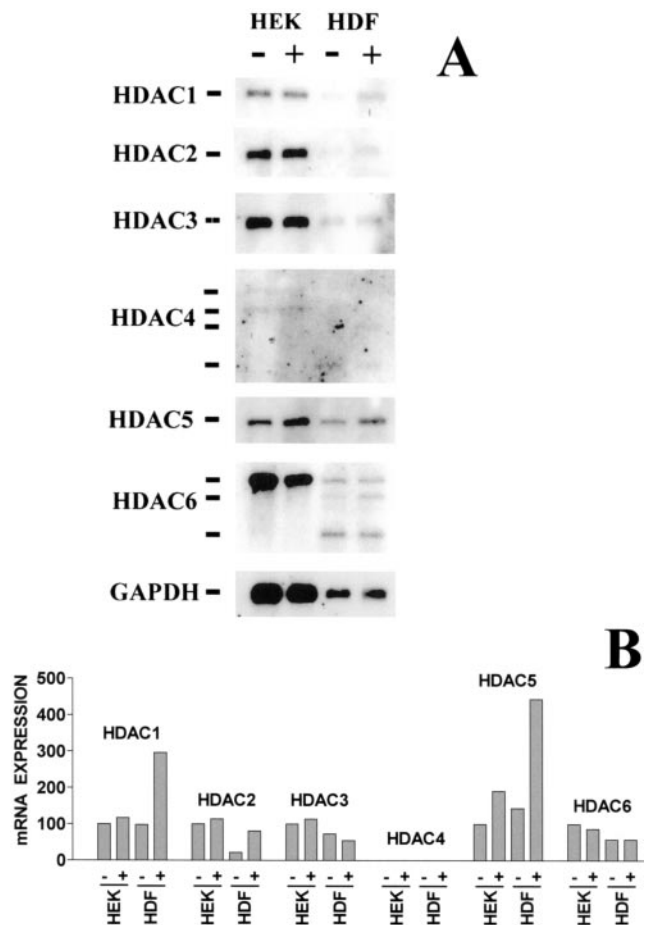
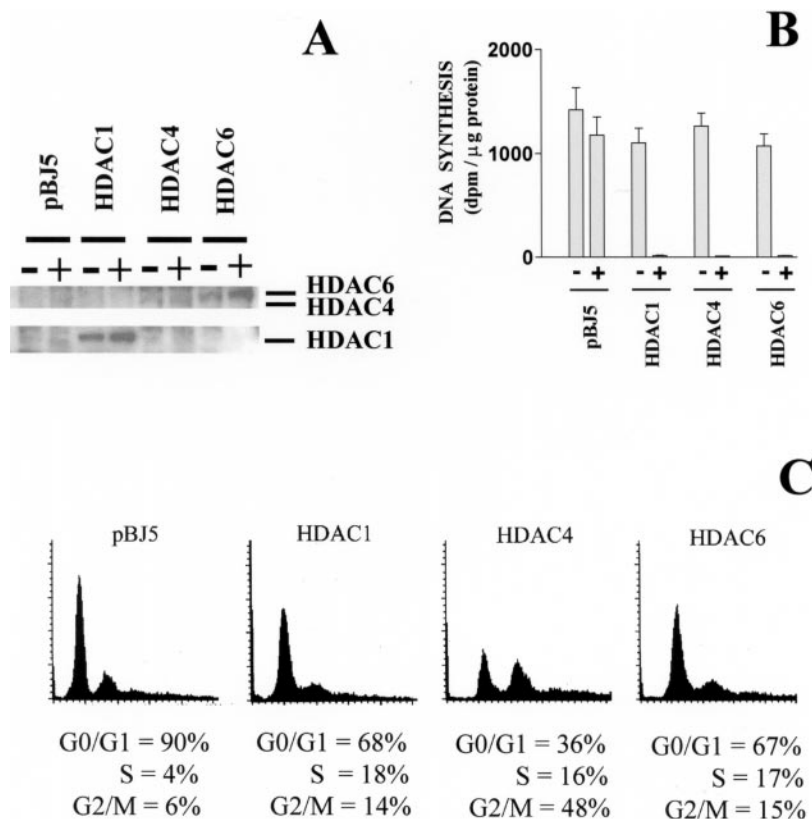


FIG. 4. mRNA expression levels for HDACs 1-6 in HEKs and HDFs. Keratinocytes (HEK) and fibroblasts (HDF) were left untreated (-) or were treated with 100 μ M SBHA for 24 h (+). Total RNA was then isolated and enriched for poly(A)⁺ RNA. One microgram of poly(A)⁺ RNA was then electrophoresed, blotted, and probed with cDNA probes specific for HDACs 1-6 or GAPDH (A). Autoradiograms were scanned with a laser densitometer and imported into Adobe Photoshop. Quantitation of mRNA expression is presented (B). Data are normalized for GAPDH expression.

by Northern analysis to determine whether the growth inhibitory response to HDAC inhibitors was associated with the expression of specific HDAC family members (HDACs 1-6; Fig. 4). There are currently eight reported HDAC family members comprising type 1 HDACs (HDACs 1-3 and 8) and type 2 HDACs (HDACs 4-7); HDAC1 (34), HDAC2 (36), HDAC3 (37, 49), HDAC4, HDAC5, HDAC6 (35), HDAC7 (50, 51), and HDAC8 (52). Analysis of HDACs 1-6 indicated that mRNA expression for the type 1 HDACs 1 and 5 were similar between untreated keratinocytes and fibroblasts when normalized for GAPDH expression (Fig. 4). In contrast, expression of HDAC2 and HDAC6 was greater in untreated keratinocytes than in fibroblasts. Furthermore, the expression of HDAC6 in HEKs was restricted to one highly expressed transcript of ~6 kilobase pairs, whereas both HDFs and SCC25 cells (data not shown) expressed very little of this transcript but did express two smaller transcripts at lower expression levels (Fig. 4). The expression of HDAC4 was difficult to assess, due to its high molecular mass (approximately 9 kilobase pairs; Ref. 35). However, keratinocytes expressed two large transcripts whereas fibroblasts expressed two smaller transcripts (Fig. 4). As with HDAC6, we did not determine whether the difference in transcripts was due to alternate spliced variants, different promoter usage, or different closely related gene transcripts. Interestingly, the expression of HDACs 1, 2, and 5 in HDFs

FIG. 5. Overexpression of HDAC1, HDAC4, and HDAC6 causes growth inhibition of fibroblasts in response to SBHA. HDFs were transfected with expression plasmids for HDAC1, HDAC4, HDAC6, or control plasmid (pBJ5). Cells were selected in G418 and then pooled clones analyzed for expression of the FLAG-tagged HDAC protein in untreated (–) or SBHA-treated (+, 100 μ M) cells (A) or growth inhibition in response to 24-h treatment with SBHA (100 μ M) (B). Data represent the mean \pm S.E. of six determinations and are presented as dpm incorporated [3 H]thymidine/ μ g of cellular protein. C, constitutive cell cycle profile of the untreated cell lines expressing the HDACs.



appeared to be induced following treatment of cells with 100 μ M SBHA for 24 h (Fig. 4). The data for HDAC mRNA expression in SCC25 cells resembled that of the fibroblasts (data not shown). Although similar amounts of poly(A)⁺ RNA were used for HEKs and HDFs, the HDFs consistently showed lower levels of GAPDH mRNA expression. This most likely reflects differences in GAPDH mRNA expression between HEKs and HDFs. Densitometric analysis in which normalization for GAPDH expression is estimated is shown in Fig. 4B.

HDAC1, HDAC4, and HDAC6 Restores SBHA-induced Growth Inhibition in HDFs—The data in Fig. 4A indicate that there are some differences in the mRNA expression levels and transcript species between HEKs and HDFs. This raises the possibility that different HDAC family members contribute to the difference in response between HEKs and HDFs. To test this we transfected HDFs with expression plasmids for the neomycin resistance gene (pSV2neo) and either pBJ5 (control vector) or plasmids coding for HDAC1, HDAC4, HDAC5, or HDAC6. Pooled colonies were enriched in media containing 500 μ g/ml G418 and then treated with vehicle or 100 μ M SBHA. Fig. 5A shows that the selected HDFs express the relevant FLAG-tagged HDAC with which they were transfected. Of the selected cells, HDAC1 appeared to have the highest expression followed by HDAC6 and then HDAC4 (Fig. 5A). HDAC5-transfected cell lines could not be established and appeared to undergo premature senescence (data not shown). Analysis of DNA synthesis in the cell lines indicated that the control selected cells were still resistant to the growth inhibitory properties of SBHA whereas HDAC1-, HDAC4-, and HDAC6-expressing cells had become profoundly sensitive to SBHA (Fig. 5B). Furthermore, the DNA synthesis between the untreated cell lines was similar, indicating that HDAC1, -4, and -6 expression did not alter constitutive proliferation of the HDFs. It was interesting to note that, although there was no difference in DNA synthesis between the untreated control and HDAC1-, HDAC4-, and HDAC6-expressing cells, there were some

marked differences in their cell cycle profile (Fig. 5C). For instance, HDAC4-expressing cells have an increased percentage of cells in the G₂/M phase (Fig. 5C). Thus, although our data revealed little change in DNA synthesis in the HDAC-expressing cells, there were profound changes in the distribution of HDAC4-expressing cells in the cell cycle, suggesting that transit through the cell cycle phases can be altered by overexpressing HDAC4. Furthermore, these data would suggest that the ability to induce growth inhibition in response to SBHA is not restricted to a particular HDAC but is a property of type 1 (HDAC1) and type 2 HDACs (HDACs 4 and 6).

Recent studies have shown that non-histone proteins are the target for acetylation/deacetylation (30). We considered the possibility that the growth inhibition of HEKs and SCC25 cells, in response to SBHA, may be associated with a hyperacetylation of proteins that were not hyperacetylated in HDFs (Fig. 6). Western blot analysis, using a generic anti-acetyllysine antibody, revealed four protein bands in HEKs and SCC25 cells that were hyperacetylated in response to SBHA and were not hyperacetylated in HDFs (Fig. 6). However, a similar analysis of HDFs overexpressing HDAC1, HDAC4, or HDAC6 did not show any corresponding hyperacetylation of proteins, in response to SBHA, that were similar to HEKs (data not shown). These data indicate that, although there are increases in proteins in HEKs and SCC25 cells, that are immunoreactive to an anti-acetyllysine antibody, following SBHA treatment, these same protein bands were not increased in HDFs or in HDFs overexpressing HDAC1, HDAC4, or HDAC6.

DISCUSSION

In the present study we have examined the ability of various HDAC inhibitors to induce histone acetylation and growth inhibition in normal keratinocytes, transformed keratinocytes, and normal fibroblasts. Our results indicate that, although all three cell types were similarly responsive to the HDAC inhibitors, with respect to the accumulation of acetylated histone

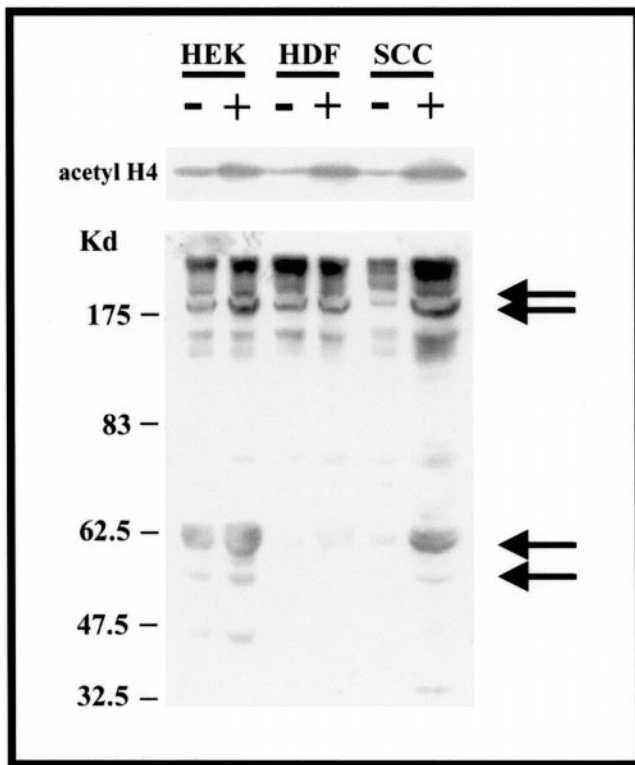


FIG. 6. SBHA causes increase in anti-acetyllysine immunoreactive bands in HEKs and SCC25 cells but not HDFs. Keratinocytes (HEK), fibroblasts (HDF), and SCC25 cells (SCC) were left untreated (–) or were treated with 100 μ M SBHA for 8 h (+). Twenty-five μ g of cellular protein was extracted, electrophoresed, blotted, and then probed for the expression of acetylated lysine. Acetylation status of histone H4 is shown at the top to verify the efficacy of SBHA. Arrows indicate immunoreactive bands that are induced in HEKs and SCC25 cells but not in HDFs.

H4, they were dissimilar in their ability to respond to the growth inhibitory properties of the HDAC inhibitors. Overall, our data provide evidence that the action of specific HDACs and HDAC inhibitors is complex and may involve mechanisms in addition to histone acetylation.

Histone H4 Acetylation Is Not Sufficient to Cause HDAC Inhibitor-mediated Growth Inhibition—There is a growing body of evidence suggesting that HDAC inhibitor-induced growth inhibition may be independent of the effects of the HDAC inhibitors on global histone acetylation status (30). Clearly, our data with HDFs indicate that increases in histone H4 acetylation status can occur in response to HDAC inhibitors in the absence of growth inhibition. Further support for the discordance between HDAC inhibitor-induced histone hyperacetylation and growth inhibition can be seen in cells in which both growth inhibition and histone hyperacetylation occur. For instance, the accumulation of acetylated histones, induced by HDAC inhibitors, in keratinocytes, transformed keratinocytes (21), and colon carcinoma cells (53) is transient. Moreover, in keratinocytes, the time at which growth inhibition occurs is not associated with hyperacetylation of histone H4 (21). Although it remains a formal possibility that the transient hyperacetylation of histones that precedes growth inhibition is sufficient to initiate growth inhibition, it is unlikely because HDAC inhibitor-induced growth suppression requires the continued presence of inhibitor at a time at which histone H4 acetylation has returned to basal levels (21). A similar finding for HT-29 cells has also been reported (15). These data indicate that the temporal events associated with histone hyperacetylation and growth inhibition, in response to HDAC inhibitors, are not

consistent with histone acetylation being the sole mediator of growth inhibition. Furthermore, we show that SBHA-induced H4 acetylation in normal fibroblasts is maximal at a dose that is not associated with growth inhibition. A similar lack of correlation between histone acetylation and growth inhibition has been reported previously for fibroblasts (18, 20), murine erythroleukemia cells (22), and MCF-7 cells (54) treated with HDAC inhibitors. Thus, HDAC-mediated growth inhibition in HDFs and HEKs has been shown to be temporally and kinetically independent of histone acetylation status.

Although it may be argued that the genes responsible for inhibiting growth in response to HDAC inhibitors are “switched off/silenced” in fibroblasts, this would seem unlikely since the overexpression of HDACs 1, 4, and 6 in fibroblasts is able to render the cells sensitive to growth inhibition by SBHA. This is also supported by the lack of further acetylation in the presence of excess HDAC inhibitor in HDFs. This would argue that all histone H4 that could be hyperacetylated has been in the HDFs. Finally, the ability of overexpressed HDACs 1, 4, or 6 to render the HDFs sensitive to growth inhibition by SBHA indicates that the ability to induce growth suppression is not restricted to specific HDACs or HDAC types.

If histone hyperacetylation is not sufficient to cause HDAC inhibitor-induced growth arrest, what are the targets of HDAC inhibitor action that contribute to growth inhibition? One possibility is that HDAC inhibitors have non-histone targets such as structural proteins (30), transcription factors (26–28, 55, 56), or cell cycle regulators (57). Attempts to identify possible non-histone protein targets by comparing acetyllysine profiles for HEKs, HDFs, and SCC25 cells, using a generic anti-acetyllysine antibody, indicated that there are non-histone proteins that are hyperacetylated in response to SBHA in HEKs and SCC25 cells that are not hyperacetylated in HDFs. Although these data provide tantalizing evidence of potential HDAC targets, it will require more rigorous analysis to determine their role, if any, in mediating SBHA-induced growth inhibition. Despite this, our data clearly show that the regulation of any alternative regulatory pathway(s), mediating HDAC inhibitor affects, clearly requires HDACs since overexpressed HDACs 1, 4, and 6 reinstate growth inhibitor sensitivity to HDFs. Although these observations do not preclude a role for histone acetylation in HDAC-mediated growth inhibition, they do show they are not sufficient to induce growth inhibition in HDFs.

Anti-proliferative Effects of HDAC Inhibitors Is Mechanistically Different between Normal Keratinocytes and Transformed Keratinocytes—The present study has demonstrated that the mechanism by which HDAC inhibitors induce growth inhibition is cell type-specific and possibly transformation-specific. For instance, SBHA treatment caused an accumulation of normal keratinocytes in the G₀/G₁ phase of the cell cycle, which was associated with an induction of the cyclin-dependent kinase inhibitor, p21 and an accumulation of hypophosphorylated pocket proteins pRb and p107. In transformed keratinocytes (SCC25 cells), SBHA treatment caused an accumulation of cells in the G₂/M phase and non-apoptotic cell death, which was also associated with an induction of p21 but no accumulation of hypophosphorylated pocket proteins pRb or p107. In contrast, human dermal fibroblasts were not growth-inhibited by SBHA yet still induced an increase in p21 expression. The induction of p21 by SBHA in HDFs was unexpected since it was not associated with reduced DNA synthesis. A similar finding has been reported by others (14). Furthermore, the p21 induced in HDFs, in response to SBHA, appeared to be inactive since it was not associated with a cell cycle block or an accumulation of hypophosphorylated pocket proteins. This suggests that p21 may not be a universal marker of HDAC inhibitor-mediated

actions and is consistent with the proposition that the response to HDAC inhibitors is cell type-specific (58). In this respect, it is important to note that epithelial cells are capable of undergoing irreversible growth arrest and terminal differentiation whereas fibroblasts generally undergo a reversible growth arrest and are not thought to undergo terminal differentiation. Given the very different biological fates of epithelial *versus* mesenchymal cells, it is not unexpected that they may respond to similar stimuli in different ways.

The cell type specificity of action of the HDAC inhibitors on the cell cycle may help to explain the seemingly paradoxical role of HDACs and HDAC inhibitors in regulating the p21/Rb/E2F axis. For instance, p21 is known to inhibit phosphorylation of Rb, suppress E2F activity, and inhibit proliferation. HDACs repress p21 transcription (45–47, 59), and release of this repression by HDAC inhibitors leads to induction of p21 and growth inhibition (4, 15). Conversely, HDAC inhibitors have been reported to relieve Rb-mediated E2F inhibition, resulting in E2F activation (23–25) and hence proliferation. Clearly, these effects of HDAC inhibitors upon p21 transcription and Rb activity oppose one another. This suggests that the biological consequences of HDAC inhibitor treatment may in fact represent the sum of these two, or more, opposing actions, which in turn may explain the differing biological outcomes for HEKs, HDFs, and SCC25 cells treated with HDAC inhibitors. For instance SBHA-mediated p21 induction in HEKs is associated with pocket protein hypophosphorylation and growth arrest, whereas in SCC25 cells, which have defective Rb/E2F regulation (31, 38), p21 induction is associated with an accumulation in G₂/M. In contrast, p21 induction in HDFs is not associated with hypophosphorylation of pocket proteins or cell cycle block, suggesting that a higher level of proliferative regulation predominates.

Possible Mechanisms for HDAC Inhibitor Action in Normal Keratinocytes, Fibroblasts, and Transformed Keratinocytes—The lack of an effect on constitutive DNA synthesis by overexpressed HDACs suggests that in normal HDFs the activity of HDACs may be subordinate to preexisting pathways controlling proliferation. Although the identity of such pathways is unknown, it is possible to speculate on the nature of such pathways. Thus, one could speculate on the existence of a regulatory framework in normal fibroblasts (or other cells) that is controlled by the location or interaction of HDACs with other regulatory proteins such that HDAC inhibitors have little effect on proliferation. When these processes are overwhelmed through the overexpression of HDACs, the normal regulatory interactions are saturated leading to the availability of HDACs to interfere with cell cycle progression. This notion gains further support from the observation that overexpression of HDAC4 is able to disrupt the constitutive cell cycle phases in fibroblasts, resulting in an equivalent fraction of cells existing in the G₀/G₁ phase as in the G₂/M phase. This proposition also is supported by the growth inhibition in HDFs overexpressing HDACs 1, 4, and 6 following treatment with SBHA. Moreover, recent reports suggest that the activity and subcellular localization of HDACs may be critical events in regulating differentiation control (60, 61).

The profound growth arrest of fibroblasts overexpressing HDACs 1, 4, and 6 in response to SBHA is consistent with a process in which proliferation control by HDACs and HDAC inhibitors in normal HDFs is selectively suppressed by homeostatic growth pathways. This implies that certain cells may have an intrinsic resistance to HDAC inhibitor-induced growth inhibition dependent upon the relative activity of these regulatory pathways. Such a proposal is not unprecedented since the discovery of interactions of HDACs with key cellular regu-

lators (58, 60–62) associated with growth and differentiation control, which indicate that the available “active” HDACs may be regulated by their interaction with, or sequestration by, other intracellular effector molecules (60, 62). For instance, HDACs are known to interact with E2F1 (26), p53 (27, 28), 14-3-3 (58, 62), MEF2A (55), Sp1 (56), ERK 1/2 (61), or Hus1/Rad9 (57). These disparate partner proteins reflect the growing complexity of our understanding of how HDACs may work and how HDAC-dependent effects may be determined by non-histone protein interactions. These data also establish that histone acetylation alone may not be sufficient to mediate HDAC inhibitor-induced actions.

Acknowledgments—We thank Dr. S. Schreiber for the generous gift of expression plasmids for HDAC1, HDAC4, HDAC5 and HDAC6. We also thank Dr. E. Seto for the generous gift of expression plasmids coding for HDAC2 and HDAC3.

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J. Biol. Chem. 2001, 276:22491-22499.

doi: 10.1074/jbc.M100206200 originally published online April 13, 2001

Access the most updated version of this article at doi: [10.1074/jbc.M100206200](https://doi.org/10.1074/jbc.M100206200)

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